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Na,K-ATPase mutations in familial hemiplegic migraine lead to functional inactivation

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Abstract

The Na,K-ATPase is an ion-translocating transmembrane protein that actively maintains the electrochemical gradients for Na^+ and K^+ across the plasma membrane. The functional protein is a heterodimer comprising a catalytic α-subunit (four isoforms) and an ancillary β-subunit (three isoforms). Mutations in the α_2-subunit have recently been implicated in familial hemiplegic migraine type 2, but almost no thorough studies of the functional consequences of these mutations have been provided. We investigated the functional properties of the mutations L764P and W887R in the human Na,K-ATPase α_2-subunit upon heterologous expression in *Xenopus* oocytes. No Na,K-ATPase-specific pump currents could be detected in cells expressing these mutants. The binding of radiolabelled [^{3}H]ouabain to intact cells suggested that this could be due to a lack of plasma membrane expression. However, plasma membrane isolation showed that the mutated pumps are well expressed at the plasma membrane. $^{86}Rb^+$-flux and ATPase activity measurements demonstrated that the mutants are inactive. Therefore, the primary disease-causing mechanism is loss-of-function of the Na,K-ATPase α_2-isoform.

Keywords: Human Na^+/K^+-ATPase α_2-subunit; ATP1A2; Familial hemiplegic migraine type 2; Pump current; Surface expression; ATP hydrolysis

1. Introduction

Na,K-ATPase belongs to the family of P-type ATPases, a major class of primary active membrane transport proteins, so called because they become transiently phosphorylated upon ATP hydrolysis. The enzyme utilizes the free energy of ATP hydrolysis to export 3 Na^+ ions and import 2 K^+ ions per ATP molecule, thereby maintaining the electrochemical gradients of Na^+ and K^+ across the plasma membrane. The Na,K-ATPase consists of a catalytic α-subunit and an accessory β-subunit. The human genome encodes four different α-subunit and three different β-subunit genes. Recently, De Fusco et al. identified two mutations (L764P and W887R) in the Na,K-ATPase α_2-subunit associated with familial hemiplegic migraine type 2 (FHM2) [1]. This important breakthrough was rapidly followed by four reports in which 11 new FHM2 mutations in the Na,K-ATPase α_2-subunit were identified [2–5], which in some cases coincided with additional clinical symptoms. The only functional data available indicates that the L764P and W887R mutants unlike the wild-type do not support cell survival [1]. It is obvious that the mutants must be functionally impaired, but it is unknown which functions are affected. We measured the functional properties of these mutants upon heterologous expression in *Xenopus* oocytes. The mutated pumps were well expressed at the plasma membrane, but no specific pump currents could be detected. In addition these mutants did not transport $^{86}Rb^+$ or hydrolyse ATP.

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2. Materials and methods

2.1. cDNA constructs

Human Na,K-ATPase α_2-subunit cDNA was subcloned into a modified pCDNA3.1 vector (Invitrogen), which additionally contained the 5′- and 3′- untranslated regions of the *Xenopus* β-globin gene flanking the multiple cloning site (kind gift of Dr. Renate Gauss, IonGate Biosciences GmbH, Frankfurt/M.). To distinguish the activity of the heterologously expressed Na,K-ATPase from the endogenous oocyte Na,K-ATPase, the mutations Q116R and N127D were introduced to obtain a ouabain-resistant protein with an IC_{50} in the mM range [6]. The ouabain-sensitive wild-type α_2-subunit construct is referred to as hNaKα_2-OuaS, the ouabain-resistant form as hNaKα_2-OuaR, the β_1-subunit as hNaKβ_1. Site-directed mutagenesis was carried out by recombinant PCR techniques. All PCR-derived fragments were verified by sequencing.

2.2. cRNA synthesis and oocyte treatment

After the appropriate linearization of plasmids, cRNA synthesis was carried out with the T7 mMessage mMachine kit (Ambion, Austin, TX). Individual stage V–VI oocytes were obtained by partial ovarectomy from anesthesized *Xenopus laevis* females, followed by collagenase treatment. For heterologous expression in oocytes each cell was injected with 15–25 ng α_2-subunit and 2.0–2.5 ng of β_1-subunit cRNAs. Cells were stored in modified Ringer's solution (90 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 5 mM MOPS, pH 7.4) containing 1 mg/ml penicillin/streptomycin at 18 °C and used for experiments 3–4 days post-injection.

2.3. Electrophysiology

Preceding electrophysiological experiments cells were placed in LS-buffer (110 mM NaCl, 2.5 mM Na-citrate, 10 mM MOPS/TRIS, pH 7.4) for 45–60 min to elevate the intracellular Na^+ concentration [7]. Afterwards cells were stored in K^+-free PL-buffer (100 mM NaCl, 1 mM $CaCl_2$, 20 mM TEACl, 5 mM $BaCl_2$, 5 mM $NiCl_2$, 5 mM MOPS/TRIS, pH 7.4) until use. Stationary currents of the Na,K-ATPase were recorded with the two-electrode voltage clamp configuration using a CA-1B amplifier (Dagan Corp., Minneapolis, MN) and pClamp 8 software (Axon Inst., Union City, CA). Data analysis and presentation was carried out with Origin 5.0 (Microcal Software, Northampton, MA).

2.4. Experimental solutions (contents in mM)

NMG-buffer (100 NMG·Cl, 5 $BaCl_2$, 5 $NiCl_2$, 5 MOPS/TRIS, pH 7.4), Na-buffer (100 NaCl, 5 $BaCl_2$, 5 $NiCl_2$, 5 MOPS/TRIS, pH 7.4), Na–K-buffer (90 NaCl, 10 KCl, 5 $BaCl_2$, 5 $NiCl_2$, 5 MOPS/TRIS, pH 7.4) were used. Stationary Na^+/K^+ pump currents of the heterologously expressed α_2-subunit were measured as the K^+-stimulated stationary current upon a solution exchange from Na-buffer to Na–K-buffer at a −40 mV holding potential. To test for specificity of the signal, cells were subsequently perfused with Na–K-buffer containing 10 mM ouabain, which blocks more than 90% of the Na^+/K^+ pump current of the heterologously expressed pump. All buffers contained 10 μM ouabain to inhibit the endogenous Na,K-ATPase of the oocytes. To test for electrogenic Na^+/Na^+ exchange currents cells were first perfused with NMG-buffer, then with Na-buffer followed by Na-buffer containing 10 mM ouabain. Neither the wild-type enzyme nor the L764P or W887R mutant showed ouabain-sensitive difference currents under these conditions. All experiments were carried out at room temperature.

2.5. Isolation of total and plasma membranes

For the isolation of total membranes, 10–20 oocytes were homogenized in 100–200 μl buffer (250 mM sucrose, 2 mM EDTA, and 25 mM HEPES/Tris (pH 7.0)) and centrifuged for 3 min at 1000×*g* and 4 °C. Next, the membranes were isolated by centrifugation of the supernatant for 30 min at 16,000×*g* and 4 °C. The isolation of plasma membranes was carried out as described previously [8,9]. Oocytes were stripped from their follicle membrane and silica was bound to the plasma membranes. After washing the oocytes were homogenized and the plasma membranes isolated by centrifugation.

2.6. Western blotting

The membrane fraction of *X. laevis* oocytes were solubilized in SDS-PAGE sample buffer, separated on SDS gels containing 10% acrylamide according to Laemmli [10] and blotted on immobilon polyvinylidene-difluoride membranes (Milipore Co., Bedford, MA). The α-subunits of Na,K-ATPase were detected with the polyclonal antibody C356-M09 [9]. The polyclonal antibody raised against the HERED sequence of Na,K-ATPase was used to probe specifically the α_2-subunit of the Na,K-ATPase [11].

2.7. [^{3}H]ouabain binding

Three days after injection the amount of heterologously expressed human Na,K-ATPase molecules in the plasma membrane of oocytes was determined, as described previously [12]. Na^+-loaded cells were incubated in K^+-free ORI buffer (contents in mM: 110 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES, pH 7.6) containing 2.5 μM [^{3}H]ouabain (17 Ci $mmol^{-1}$=6.29×10^{11} Bq $mmol^{-1}$), 2.5 μM unlabeled ouabain and 1 mM sucrose (with 18 μM [^{14}C]sucrose=14.8 kBq/200 μl) for 20 min at room temperature followed by 5 washes in ouabain-free buffer. Since the plasma membrane

of intact oocytes is virtually impermeable to sucrose, the inclusion of radiolabelled sucrose in the incubation buffer allows to unambiguously distinguish unspecific inward leakage of ouabain from ouabain binding to the plasma membrane. Ouabain-binding data from cells with increased [^{14}C]sucrose uptake (>10% of the detected [^{3}H]ouabain signal) were rejected. After solubilization of individual cells with 100 μl 5% (w/v) SDS buffer radioactivity was counted by liquid scintillation analysis.

In addition [^{3}H]ouabain binding to total membranes was measured. A total membrane fraction equivalent to 2.5 oocytes was incubated at room temperature in 50 mM Tris–acetic acid (pH 7.0), 1 mM H_3PO_4, 5.0 mM $MgCl_2$ and 250 nM [^{3}H]ouabain (17 Ci mmol^{-1}=6.29×10^{11} Bq mmol^{-1}) in a final volume of 50 μl. After 1 h the reaction mixture was chilled for 15 min at 0 °C. The ouabain–protein complex was collected by filtration over 0.8 μm membrane filter (Schleicher and Schuell, Dassel, Germany). After washing twice with ice-cold water, the filters were analyzed by liquid scintillation analysis.

2.8. $^{86}Rb^{+}$ flux measurements

The uptake of radiolabeled Rb^{+} into oocytes was measured as follows: Oocytes loaded with Na^{+} as described above were pre-incubated for 10 min in K^{+}-free buffer BBB (in mM: 90 TMA·Cl, 20 TEA·Cl, 10 $BaCl_2$, 5 $NiCl_2$, 5 MOPS, pH 7.4) containing either 100 μM or 10 mM ouabain, and then placed for 12 min in flux buffer (in mM: 88.2 TMA·Cl, 19.6 TEA·Cl, 9.8 $BaCl_2$, 4.9 $NiCl_2$, 4.9 MOPS, 5 mM RbCl, 0.01 μCi μl^{-1} $^{86}Rb^{+}$ (=6.9×10^{5} Bq ml^{-1}), pH 7.4) containing either 100 μM or 10 mM ouabain. After four washes in Rb^{+}-free buffer BBB, cells were individually placed into counting vials and analyzed by liquid scintillation analysis. The procedure was carried out at room temperature (21 °C).

2.9. ATPase measurements

Membranes of injected *Xenopus* oocytes (obtained from 15–20 cells) were added to a medium containing 50 mM TRIS–acetic acid (pH 7.0), 0.2 mM EDTA, 0.1 mM EGTA, 1 mM TRIS–N_3, 1.3 mM $MgCl_2$, 100 mM NaCl, 100 μM [γ^{32}P]ATP (3000 Ci mmol^{-1}), with and without 10 mM KCl. After incubation at 37 °C, the reaction was stopped by the addition of 500 μl 10% (w/v) charcoal in 6% (w/v) trichloroacetic acid. The mixture was centrifuged for 30 s (10,000×g) and the supernatant, containing the liberated inorganic phosphate ($^{32}P_i$), was analyzed by liquid scintillation analysis.

2.10. Calculations

Data are presented as mean values with standard error. Differences were tested for significance by means of the two-tailed Student's t test on independent samples.

2.11. Materials

[^{3}H]ouabain, [^{14}C]sucrose, $^{86}Rb^{+}$, and [γ-^{32}P]ATP were purchased from Amersham Pharmacia (Buckinghamshire, United Kingdom).

3. Results

To investigate the structural basis of the mutations leading to FHM2, a molecular model of the Na,K-ATPase α_2-subunit was built using the E_2 structure of the sarcoplasmic reticulum Ca^{2+}-ATPase [13] as a template (Fig. 1). The mutated residue Leu-764 resides within the intracellular portion of the ~70 Å long α-helical M5 segment, which is a crucial element of the 3D structure. This α-helix contributes on one side to the cation binding pocket within the transmembrane region and on the other side extends into the catalytic domain involved in ATP hydrolysis and intermediate phosphorylation. As several research groups demonstrated conformational flexibility of the M5 segment during the catalytic cycle, this segment

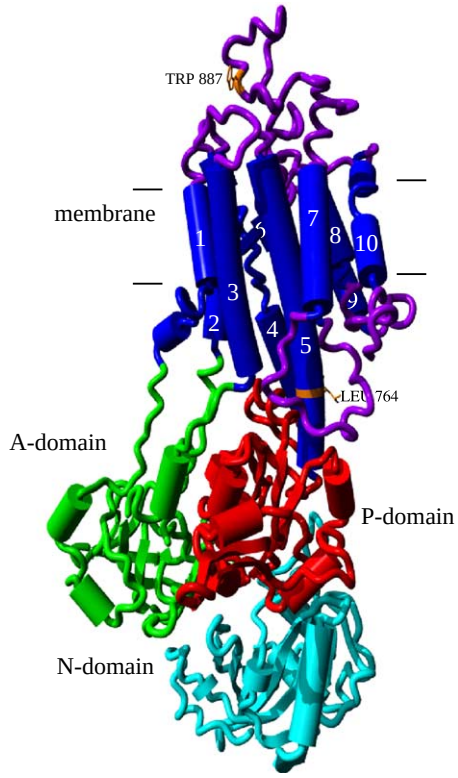

Fig. 1. Location of residues Leu-764 and Trp-887 in the Na,K-ATPase α_2. The Na,K-ATPase α_2-subunit amino acid sequence was modelled into the 3D structure of the Ca^{2+}-ATPase from sarcoplasmic reticulum in the E_2-conformation using Swiss-model [33]. The image was created with YASARA (www.yasara.org). Leucine 764 resides within the intracellular portion of the ~70 Å long α-helical M5 segment. Tryptophan 887 is located in the extracellular loop between transmembrane segments M7 and M8.

is suggested to play an important role in the energy transduction mechanism that couples the ATP hydrolysis to cation transport. Residue Trp-887 is located in the extracellular loop between transmembrane segments M7 and M8, which is implicated in the α–β intersubunit interaction [14,15]. Since Ca^{2+}-ATPase does not contain a β-subunit, modeling on basis of the Ca^{2+}-ATPase structure is not meaningful for the W887R mutation.

To test for the effects of the L764P and W887R mutations on Na^+/K^+ pump activity, ouabain-resistant wild-type and mutant human Na,K-ATPase α_2-subunits (each together with the human β_1-subunit) were expressed in *Xenopus* oocytes and stationary currents in response to 10 mM extracellular K^+ were measured in two-electrode voltage-clamp experiments (Fig. 2). To distinguish the activity of the heterologously expressed enzyme from the endogenous Na,K-ATPase of the oocytes all perfusion buffers contained 10 μM ouabain, which blocks the endogenous high-affinity pump. Fig. 2A shows typical recordings of stationary currents of the wild-type human Na^+/K^+ pump in response to a solution exchange from zero to 10 mM K^+. The specificity of the signal is demonstrated by the inhibitory action of 10 mM ouabain, which blocks more than 90% of the stationary current of the ouabain-resistant wild-type α_2-subunit. No K^+-stimulated, ouabain-sensitive currents were observed in cells expressing the L764P or W887R mutants, as summarized in Fig. 2B. Whereas the hNaKα_2-OuaR construct generated an average K^+-stimulated current level of 103±28 nA, the currents for the hNaKα_2-OuaR(L764P) and hNaKα_2-OuaR(W887R) mutants (0±2 nA and 4±4 nA, respectively) were not significantly different from zero. As it has been reported that some Na,K-ATPase mutations can lead to a high level of ATP-driven, electrogenic Na^+/Na^+ exchange activity [16] we also tested for ouabain-sensitive (10 mM) currents in the absence of K^+. No such activity could be detected for the wild-type enzyme or for both FHM2 mutants (data not shown).

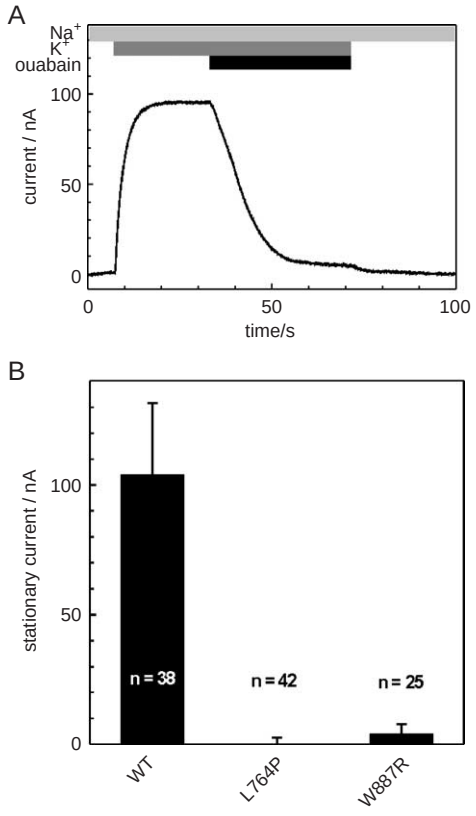

Fig. 2. Stationary currents of wild-type human Na,K-ATPase and mutants L764P and W887R expressed in *Xenopus* oocytes. (A) Stationary currents recorded on oocytes expressing the hNaKα_2-OuaR (plus hNaKβ_1) construct (upper panel) upon solution exchanges from Na-buffer to Na–K-buffer and Na–K-buffer plus 10 mM ouabain, as indicated by the perfusion scheme above the signal traces. Holding potential was −40 mV. (B) Mean stationary current amplitudes (stimulated by 10 mM K^+ at −40 mV) from oocytes expressing hNaKα_2-OuaR wild-type (WT) and mutants hNaKα_2-OuaR(L764P) (LP) and hNaKα_2-OuaR(W887R) (WR). Data were obtained from 3 different oocyte preparations (mean±standard error).

To test whether the lack of pump current was due to the amount of protein at the plasma membrane of oocytes, the binding of radioactively labelled [^{3}H]ouabain to oocytes expressing the Na,K-ATPase α- and β-subunits was measured (Fig. 3A). For these experiments the ouabain-sensitive constructs (wild-type hNaKα_2-OuaS or the mutants hNaKα_2-OuaS(L764P) or hNaKα_2-OuaS(W887R)) had to be used. As the endogenous oocyte Na,K-ATPase, which is also ouabain-sensitive, contributes to the total signal, ouabain binding was also measured on non-injected control oocytes to quantify this background. Since it has been reported that β-subunit expression alone stimulates plasma membrane expression of the endogenous Na,K-ATPase in oocytes [12,17], we established quantitatively that expression of hNaK-β_1 alone hardly increased the plasma membrane expression of the endogenous pump (data not shown). Fig. 3A shows that ouabain binding to hNaKα_2-OuaS(WT)/hNaK-β_1 expressing oocytes was almost 4 times larger than in non-injected controls. However, in contrast to the wild-type protein the expression of both FHM2 mutant α_2-subunits did not lead to increased ouabain binding beyond the level found for expression of the hNaK-β_1 construct alone.

If the two mutant enzymes would not be expressed at the plasma membrane, their presence within cells can be proven by [^{3}H]ouabain binding to the total membrane fraction. Fig. 3B shows the ouabain binding of the wild-type hNaKα_2-OuaS and the mutants hNaKα_2-OuaS(L764P) and hNaKα_2-OuaS(W887R). The binding capacity of L764P and W887R is not significantly different from that of non-injected oocytes. This indicates that the mutants are either not expressed or have a drastically lower affinity for ouabain.

To rule out the alternative that the absence of detectable pump currents or ouabain binding is due to reduced protein expression levels, Western blot analysis was carried out

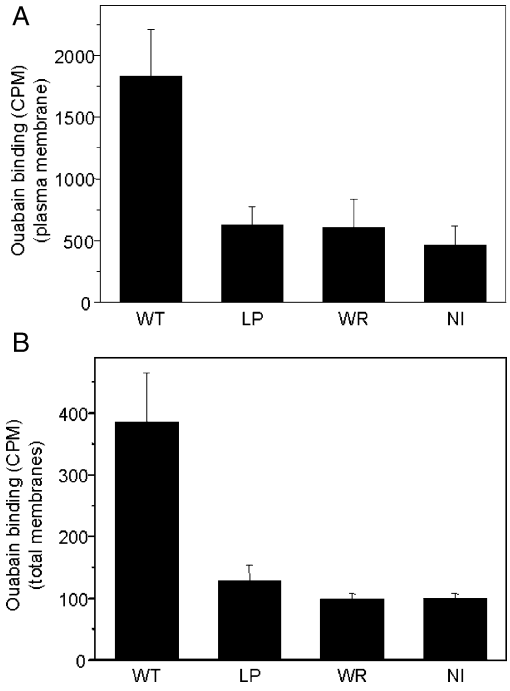

Fig. 3. [^{3}H]ouabain-binding to oocytes and the isolated total membranes of oocytes expressing ouabain-sensitive Na,K-ATPase constructs. Oocytes were co-injected with hNaKα_2-OuaS/hNaK-β_1 (WT), hNaKα_2-OuaS(L764P)/hNaK-β_1 (LP) and hNaKα_2-OuaS(W887R)/hNaK-β_1 (WR) cRNAs, respectively. As the endogenous Na,K-ATPase of the oocytes contributes to high-affinity ouabain binding, the background due to the endogenous pump was determined by measuring not-injected control oocytes from the same preparation. Panel (A) shows the [^{3}H]ouabain binding to the plasma membrane of intact oocytes (9–15 cells for each column), whereas panel (B) shows the [^{3}H]ouabain binding to the isolated total membranes (data from 4 independent experiments). Values are means±standard errors.

using the C356-M09 antibody that was directed against the Na,K-ATPase α_1-subunit, but also recognizes the Na,K-ATPase α_2-subunit. In addition we used the anti-HERED antibody that specifically recognizes the Na,K-ATPase α_2-subunit [11]. Total membranes and plasma membranes were isolated from oocytes co-injected with cRNA for hNaKα_2-OuaR wild-type and both FHM2 mutants each together with hNaK-β_1 cRNA. Uninjected oocytes from the same preparations were used as controls. Western blotting of multiple samples showed that the expression of the L764P and the W887R mutant was similar to that of the wild-type (Fig. 4A). The samples isolated from the plasma membranes (Fig. 4B) show that both mutants and the wild-type enzyme are present at the plasma membrane in similar amounts. To check if the plasma membrane isolation technique is specific in our hands, we expressed aquaporin-2 (AQP2) wild-type and an AQP2 mutant (T126M) in *X. laevis* oocytes. As was reported by Mulders et al. [18], we observed that the wild-type is expressed in the plasma membrane whereas the mutant is not expressed in the plasma membrane under these conditions (data not shown). The weak signal seen in the total membrane fraction with antibody C356-M09 is probably not an endogenous Na,K-ATPase α-subunit, because it is not present in the plasma membrane fraction.

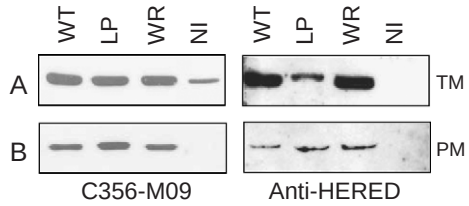

Fig. 4. Determination of Na^+/K^+-ATPase expression in total membrane and plasma membrane preparations of *X. laevis* oocytes. Western blot analysis of total membranes (A) and plasma membranes (B) from not-injected oocytes (NI) and oocytes expressing hNaKα_2-OuaR wild-type (WT), hNaKα_2-OuaR(L764P) (LP) mutant, and hNaKα_2-OuaR(W887R) (WR) mutant (each plus hNaKβ_1). Detection was carried out using a polyclonal antibody that recognizes different Na,K-ATPase α-subunits (C356-M09) and a polyclonal antibody specifically directed against the Na,K-ATPase α_2-subunit (anti-HERED). TM denotes total membranes, PM plasma membranes.

Obviously the mutant enzymes are expressed at the plasma membrane, but are neither able to bind ouabain nor to sustain electrogenic pump activity.

To explore the possibility that the lack of K^+-induced stationary currents in the case of mutants L764P and W887R might be due to a change in the Na^+/K^+ stoichiometry of transport towards electroneutral exchange of cations, $^{86}Rb^+$ tracer flux measurements were carried out. As shown in Fig. 5, the expression of the wild-type Na,K-ATPase α_2-subunit led to a 20-fold increase in $^{86}Rb^+$ uptake over uninjected control oocytes. In contrast, both constructs carrying FHM2 mutations exhibited a drastic reduction in Rb^+ uptake. In case of the L764P mutant Rb^+ flux was not significantly different from that observed in non-injected control cells.

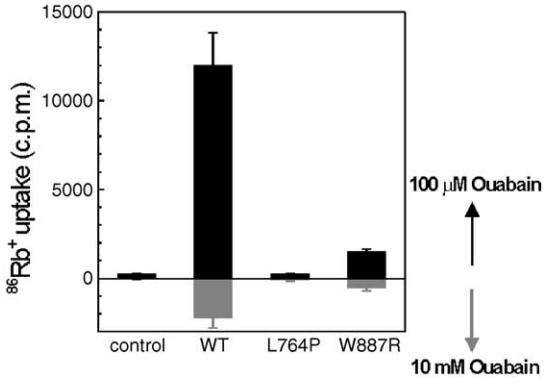

Fig. 5. $^{86}Rb^+$ tracer flux measurements. Oocytes were coinjected with hNaKα_2-OuaR/hNaK-β_1 (WT), hNaKα_2-OuaR(L764P)/hNaK-β_1 (L764P) and hNaKα_2-OuaR(W887R)/hNaK-β_1 (W887R) cRNAs, respectively, and individually assayed for $^{86}Rb^+$ uptake by liquid scintillation analysis. To dissociate the activity of the heterologously expressed Na,K-ATPase from that of the endogenous pump, ouabain-resistant constructs were expressed. $^{86}Rb^+$ uptake was performed either in a medium containing 100 μM ouabain, which only inhibits the endogenous pump (black bars, pointing upwards), or in a 10 mM ouabain medium, which largely abolishes the activity of the heterologously expressed pump (grey bars, pointing downwards). The background due to the endogenous pump was determined by measuring the uninjected control oocytes from the same preparation either in ouabain-free (black bar) or in 100 μM ouabain medium (grey bar). Data were obtained from 9–13 oocytes from a single batch of cells (means±standard error).

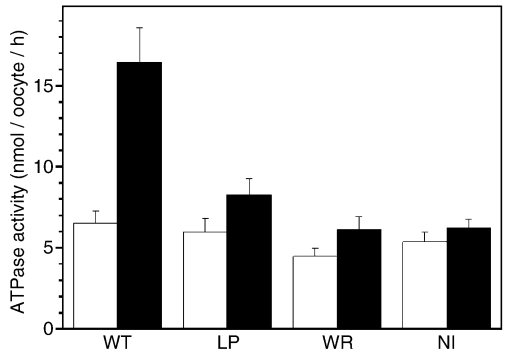

Fig. 6. ATPase activity of wild-type and mutant Na,K-ATPases. Total membranes from not injected oocytes (NI) and oocytes expressing hNaKα_2 wild-type (WT), hNaKα_2-(L764P) mutant (LP), and hNaKα_2-(W887R) mutant (WR) (each plus hNaKβ_1) were isolated. The ATPase activity was measured in the presence of 100 mM NaCl, and 100 μM ATP in the absence (white bars) or presence (black bars) of 10 mM KCl. Data were obtained from 5–6 different oocyte preparations (mean±standard error).

Mutant W887R showed a small residual Rb^+ uptake, which was, however, not significantly higher than in cells injected with β_1-subunit cRNA alone.

Finally, we tested the ATPase activity in the presence of 100 mM Na^+, 10 mM K^+ and 100 μM ATP. For this purpose the total membrane fractions of the wild-type, mutant L764P, mutant W887R, and not-injected oocytes were used. The activity measured in the absence of K^+ was about equal for all preparations (Fig. 6). The addition of K^+ stimulated the activity of the wild-type enzyme. We also detected an endogenous K^+ stimulated ATPase activity in all independent ATPase activity measurements, but this activity was very little compared to that of recombinant Na,K-ATPase activity. The activity of the two mutants did not significantly increase compared to that measured in samples from non-injected oocytes. This indicates that the two mutant enzymes are completely inactive.

4. Discussion

The functional consequences of the FHM2 mutations L764P and W887R on the expression and activity of the human Na,K-ATPase α_2-subunit were evaluated. According to the data presented here the mutations lead to a complete loss of catalytic function. No Na^+/K^+ pump currents could be measured upon heterologous expression of the mutant proteins in *Xenopus* oocytes, although it could be shown that all mutant Na,K-ATPase proteins were expressed. The amount of Na,K-ATPase at the plasma membrane was not reduced. As the mutations in humans are present in the heterozygous state, the pathogenic effect of the mutations in FHM2 affected individuals can be attributed to a severe gene dosage effect of functional Na,K-ATPase α_2-subunit in the respective tissues. One has to mention that a dominant negative effect brought about by the mutations cannot be ruled out, as there are multiple reports on dimerization of the holoenzyme (see e.g. the recent article by Laughery et al. [19]).

De Fusco et al. [1] showed that HeLa cells die within 48 h after treatment with 1 μM ouabain. If these cells are transfected with an ouabain-resistant form of the wild-type Na,K-ATPase α_2-subunit they survive. The L764P and W887R mutants (also carrying mutations which confer ouabain resistance), however, do not give cell survival. This result indicates that the mutants are functionally impaired. Although these findings are suggestive, they do not sufficiently prove that these mutations lead to a complete loss of function of the Na,K-ATPase. It might be that their transport mode deviates from the usual $3Na^+/2K^+$ stoichiometry for each hydrolyzed ATP molecule, as found for some mutated Na,K-ATPase constructs [20,21]. In addition, mutants that exhibit a large ATP-dependent electrogenic Na^+/Na^+ exchange activity have been reported [16,22,23]. Also the affinity for ouabain, cations, or ATP might be changed. The most extreme reason for the lack of cell survival would indeed be a complete loss of function. If the mutant Na,K-ATPase would not be present in the plasma membrane cell survival would also be eliminated. De Fusco and coworkers [1] reported that the mutant pumps are present in the plasma membranes. The figure showing immunocytochemical detection of the Na,K-ATPase in transfected COS-7 cells, however, does not provide definitive proof of plasma membrane expression; all visible signals seem to be present in the cytosol, probably due to overexpression. Additionally, they reported plasma membrane isolation by subcellular fractionation. However, unless marker enzymes are used to unambiguously identify membrane compartments, the composition of the obtained membrane fractions is questionable.

In the present study we demonstrated that both FHM2 mutations completely inactivate the enzyme's activity; there was no pump current, no Rb^+ uptake or ATPase activity present. In addition, we showed by plasma membrane isolation of *X. laevis* oocytes that the mutated Na,K-ATPases are present at the plasma membrane. Obviously, the mutant enzymes readily pass cellular quality control mechanisms to exit the ER and reach the plasma membrane, making an impaired protein folding highly unlikely. However, there is a structural effect of the mutations that lead to a reduced ouabain affinity since ouabain binding to the plasma membrane or total membrane fraction could not be observed. The distance between L764P and ouabain binding-sensitive residues in TM segment 5 (Phe-783 [24], Phe-786 [25], Leu-793 [26]) requires a long-range effect. The kink introduced by proline between the phosphorylation and the cation binding sites probably interferes with energy transduction. It is most likely that due to this interference the L764P mutant cannot obtain the conformation that is needed for ouabain binding. The complete loss of activity of the W887R mutant is possibly also responsible for the loss of ouabain binding function of this mutant. However, the close proximity of Arg-880 that has been shown to influence the

ouabain affinity might indicate a more direct effect of Trp-887 in the loss of ouabain binding function [27]. The mutations obviously do not interfere with the correct targeting of the enzyme. The occurrence of the α_2-subunits at the plasma membranes is also indicative of an intact interaction with the β-subunit during membrane insertion and protein folding accompanying biosynthesis in the ER, as the injection of cRNA of the α-subunit alone into oocytes does not lead to detectable amounts of the α-subunit protein at the plasma membrane [9,28–30]. The group of Fambrough [14,15] showed that four amino acids in the second half of the extracellular loop between TM segments 7 and 8 of the α_1-subunit are essential for binding to the β-subunit. Although the Trp-887 is located at the end of the first half of loop 7–8, the W887R mutation apparently does not obstruct the binding of the β-subunit. The substitution of the neutral tryptophan by a positively charged arginine residue might, however, interfere with the functional interaction between the α- and β-subunit during catalytic activity, which involves large conformational changes of the holoenzyme. Unfortunately, there is no detailed structural or time-resolved information about this interaction. But since the β-subunit is absolutely required for enzyme function, an impairment of the dynamic interaction between the subunits might involve significant changes even in the structure of the enzyme's cation binding sites.

Leucine 764 resides within the intracellular portion of the ~70 Å long α-helical M5 segment. M5 is a crucial structural element as on one side it contributes several residues to the cation binding pocket within the transmembrane region and on the other side extends into the catalytic domain involved in ATP hydrolysis and intermediate phosphorylation. A proline residue can break an α-helix. Helix M5 probably provides the interaction between the cation binding domain and the phosphorylation domain and was suggested to play an important role in energy transduction between ATP hydrolysis and ion transport [31]. The disruption of M5 would have drastic effects for the enzyme. This is exactly what occurs after the introduction of mutation L764P into the human Na,K-ATPase α_2-subunit. The complete functional inactivation observed for the two FHM2 mutants in this study differs from recent results on another FHM2 mutation (T345A, [3]), in which a shift in the apparent K^+ affinity of the mutant enzyme was reported [32]. Therefore, on a molecular level, the mechanisms which lead to the FHM2 phenotype can involve a broad spectrum of changes in enzymatic activity.

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